Appendix A

Protocols for the HET-CAM Test Method

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Appendix A1

INVITTOX Protocol 47. The HET-CAM Test – Method of Spielmann and Liebsch

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HET-CAM TEST

The potential irritancy of compounds may be detected by observing adverse changes which occur in the chorionallantoic membrane of the egg after exposure to test chemicals.

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Rationale

Chemicals are placed directly onto the chorionallantoic membrane of the hen's egg. The occurrence of vascular injury or coagulation in response to a compound is the basis for employing this technique as an indication of the potential of a chemical to damage mucous membranes (in particular the eye) *in vivo*.

Basic Procedure

Hen's eggs are rotated in an incubator for 9 days after which time any defective eggs are discarded. The shell around the air cell is removed and the inner membranes are extracted to reveal the chorionallantoic membrane. Test chemicals are added to the membrane and left in contact for 5 minutes. The membrane is examined for vascular damage and the time taken for injury to occur is recorded. Irritancy is scored according to the severity and speed at which damage occurs.

Critical Assessment

The test has several advantages including its simplicity, rapidity, sensitivity, ease of performance and its relative cheapness.

A factor to consider is the fertility and the ability of the eggs to hatch. The survival of chickens is dependent on a complex interrelationship of ecological factors (e.g. the genetic background and the age of the mated birds, the nutritional status and general management of the flock, and in part seasonal variations). Eggs should, therefore, be obtained from reliable local contractors. (The authors have produced some empirical data on the fertility of the particular flocks they use. The fertility of middle-aged flock is approximately 90% with 10-15% defective eggs. On average there are 20% lesions produced during preparation.)

The major disadvantage of the procedure is the subjective nature of the evaluation of the results. This is overcome to a certain extent by the inclusion of positive standards and by using a comprehensive scheme for scoring the irritant effects of the chemicals.

The exposure period of 5 minutes to the test chemical has been found to be sufficient to reveal irritant/toxic effects (longer exposure does not appear to yield any additional information).
A factor for consideration is whether the Hen's egg test may be considered as an animal experiment. At present the test is often looked upon as being borderline, although it has potential to be used in a manner likely to reduce the number of mammals used in conventional testing and also to contribute towards a reduction in the associated suffering.

Test Status

This test, along with several other *in vitro* systems, is presently undergoing validation as an alternative test to replace the Draize Rabbit Eye Test, in a national interlaboratory study started in June 1988, by the Federal Health Office (BGA) of the Federal Republic of Germany (FRG).

The aim of this collaborative study is to validate the classification of chemicals, with regard to their irritation potential, using the Neutral Red/ Kenacid Blue (NR/KB) cytotoxicity assay and the Hen's Egg Test Chorioallantoic Membrane (HET-CAM) assay according to Lupke. The FRG Public Health Office (BGA) is coordinating the scheme which includes, 12 toxicology laboratories in the chemical industry, universities, the BGA and other research institutions who will study 44 substances with a variety of chemical, biochemical, and toxicological characteristics. The validation test is intended to provide comparative data for the development of an alternative routine test scheme, and which is performed under routine laboratory conditions.

The validation project of alternatives for the Draize test consists of three parts: a preliminary phase, an interlaboratory assessment, and, finally, the development of a database of results. During the preliminary phase the cytotoxicity test and the HET-CAM assay have been established in the different laboratories. The participants have agreed on standard and mandatory protocols and on the choice of chemicals. Two preliminary trials have been performed with 4 test substances.

During the interlaboratory assessment 35 chemical substances of a variety of chemical structure groups have been tested with both alternative techniques in 12 laboratories under conditions that will be defined in the preliminary phase of the study. The main purpose of the validation phase is the comparative and statistical evaluation of all data at the BGA followed by a final scientific validation which could prove of interest to regulatory authorities. This assessment determines both the reproducibility of the results within a given laboratory and of a given test between laboratories.

Preliminary findings indicate that data from the HET-CAM test appears to correlate better than the two cytotoxicity tests when compared to *in vivo* Draize scores. The cytotoxicity tests give a greater number of false positives and negatives compared to the HET-CAM test. The cytotoxicity tests have, however, given better reproducibility of test data, within and between laboratories, than the HET-CAM. This is most probably due to the automated determination of NR and KB values and to the highly subjective determination of the toxicological endpoints in the HET-CAM test which are difficult to standardize. In conclusion, both the cytotoxicity tests and the HET-CAM test can provide reproducible results if carried out under routine conditions with well trained operators.

The third phase of the validation project, database development, commenced on June 1st, 1990. Seven laboratories are testing a total of 200 chemicals which again include a variety of chemical classes.

Chemicals Tested

Acetone	Lactic acid (5%)
Acetonitrile	*n*-Hexane
Acrylamide	Nicotinamide
Aniline	Nitrobenzene
Ascorbic acid	Phenol
Benzalkonium chloride (0.5%)	Propanediole
Benzoic acid	2-Propane-1-ol
2-Butoxyethanol	Pyridine
Copper(*II*) sulphate	SDS (1%)
Cyclohexanol	Sodium chloride
DEHP (100%)	Tetrachloroethane
Dimethylsulphoxide	Thiourea
EDTA-Na salt	Toluene
Ethanol	

References

Lüpke, N.P. (1986)
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Interlaboratory assessment of alternatives to the Draize eye irritation test in Germany.
Toxic. in Vitro, **5 No.5/6**, 539-542.

Procedure Details

Note: The herewith included details on the procedure have been sent to the person responsible for the method to update or confirm it. As soon as new information will become available this version will be updated.

Animal
White Leghorn chicken eggs (Shaver Starcross 288A)
The White Leghorn chicken has been selected for several reasons;
The ability to hatch the eggs of this breed is very consistent and reproducible. There does not appear to be any hereditary defects in this

breed.

Equipment

Incubator with an automatic rotating device, e.g. Ehret GmbH,
220 V, 50 Hz, 360 watt
Optimum temperature: 37.5^{0}C (± 0.5^{0}C)
Relative humidity 62.5% (± 7.5%)

Candling light
Dentist's rotating sawblade
Computer with appropriate software (HET-CAM evaluation program) - not commercially available (authors will give assistance to interested scientists)
Cold-light lamp
pH-meter
Thermometers
Tapered forceps
Pipettes (300µl application)
Stopclock

Materials

NaCl
SDS
0.1 N NaOH

Make up the following solutions:
0.9% NaCl solution in distilled water
1% SDS solution in distilled water

Test chemicals
Make up the chemicals in 0.9% NaCl solution or olive oil.

Method

N.B. Avoid any shaking, unnecessary tilting, knocking, and all other mechanical irritation of the eggs when preparing them for the assay.

Incubation of eggs
Select fresh fertile 50-60g eggs. Candle the eggs and discard any which are defective.

Place the eggs flat onto incubator trays in a 37.5^{0}C incubator and rotate for 8 days to prevent the attachment of the embryo to one side of the egg.
Check the temperature and humidity at the same time each day.
Candle the eggs on the 9th day and discard any non-viable eggs.
Replace in the incubator with the large end upwards but do not rotate, thus ensuring accessibility to the chorionallantoic membrane.
On day 10 prepare the eggs for assaying.

Assay preparation
Candle each egg to ensure that all are viable. Use cold lamp to ensure an optimal illumination of the chorioallantoic membrane.

Carry out in a fume cupboard with safety goggles to prevent inhalation and contact with the fine egg shell powder. Mark the air cell using a rotating dentist-sawblade and pare the section of the shell off.

Carefully moisten the membrane with 0.9% NaCl solution at 37^0C.

Replace eggs in incubator until ready for assaying (maximum of 30 minutes between opening the eggs and starting the assay).

Freshly prepare standards and test solution (in the appropriate solvent) before each assay at room temperature. Measure and record ph.

Assay procedure
Take the opened egg out of the incubator, pour off the 0.9% NaCl solution, carefully remove the egg membrane without injuring any underlying blood vessels using tapered forceps.

Add 0.3ml of the standard, or test chemical solution to the CAM.

Observe the reactions on the CAM over a period of 5 minutes. Monitor the appearance of:

haemorrhage (Bleedings)
vascular lysis (Blood vessel disintegration)
coagulation (protein denaturation intra- and extra vascular)

Record in seconds, the time for each reaction to occur and calculate an **irritation score (IS)**.

$$IS = \frac{301 - sec\ H}{300} \cdot 5 + \frac{301 - sec\ L}{300} \cdot 7 + \frac{301 - sec\ C}{300} \cdot 9$$

H : Haemorrhage
L : Vessel lysis
C : Coagulation
sec : Start Second

When determining the threshold the degree of severity of each reaction after treatment time has to be recorded according to the following scheme:
0 = no reaction
1 = slight reaction
2 = moderate reaction
3 = severe reaction

The threshold is then defined to be the highest concentration, at which slight reactions occur. To determine the threshold apply 0.3ml of the starting concentration (a good choice is 5% if no further information is given) to three eggs each. Graduate the severity of the main reaction after 5 minutes. If the observed reaction is slight , double the concentration. If the reaction is moderate or severe, divide the concentration by two or ten to get the next test concentration. Proceed further until the threshold concentration is found.

Test Scheme
For a given chemical the procedure consists of four steps:
1) Determine the irritation score (IS) for the two standards with two eggs

each.

N.B. 1% SDS should give an IS of 10±2 and 0.1 NaOH an IS of 15±3.

2) Determine the threshold concentration of the test chemical as described above.

3) Determine the IS for a 10% solution for three eggs. For insoluble substances take the supernatant of a standard solution.

4) Determine the IS for the pure substance (100%). If the test chemical is an insoluble solid substance, proceed as follows: Instead of determining the IS, put some grains of the substance onto the CAM to cover approximately half of its surface. After 5 minutes carefully rinse off the test material with NaCl solution and record the severity of each of the three reactions (haemorrhage, lysis, coagulation) according to:
0 = no
1 = slight
2 = moderate
3 = severe

If any reaction of degree 3 has been observed, repeat the procedure with three new eggs, rinsing after one minute.

At the end of the assay kill the embryos as quickly as possible (e.g. by placing the eggs into a freezer at -20^{0}C).

Calculations and Classifications
Calculate the mean value of the IS for the three eggs for each of the two runs and both concentrations as well as the mean over both runs of the IS and threshold concentration.

A classification of the irritating potential can be carried out according to the following (preliminary) classification scheme.

Threshold (TH) concentration	Irritation score (10%)	Severity	Classification
TH < 1%			severe/corr
1.0 < TH < 2.5	> 16		severe/corr.
2.5 < TH < 10.0	< 16	severe reaction after 1 min.	severe/corr.
1.0 < TH < 2.5	< 16		irritant
2.5 < TH < 10.0	> 16		irritant
2.5 < TH < 10.0	< 16	severe reaction after 5 min	irritant
2.5 < TH < 10.0	< 16	weak or no reaction	moderate
10.0 < TH	> 16		moderate
10.0 < TH	< 16	severe reaction	moderate
10.0 < TH	< 10		no/slight

Experimental Data

Preliminary results of the interlaboratory study are published in:
Spielmann, H. *et al.* (1991): Interlaboratory assessment of alternatives to the Draize eye irritation test in Germany. *Toxic. in Vitro*, **5 No. 5/6** , 539-542.

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Appendix A2

Table of HET-CAM Protocols from the Reviewed Literature

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	Luepke 1985	Luepke and Kemper (1986)	Kalweit et al. (1987)	Reinhardt et al. (1987)	Kalweit et al. (1990)
Hen Strain	White Leghorn (Shaver Starcross 288A, Lohmann Selected Leghorn LSL)	Lohmann Selected Leghorn, LSL	White Leghorn	Not Noted	White Leghorn
Egg Criteria for Use	Prior to use, eggs are candled to remove defective eggs. Eggs weighing < 50 g or > 60 g are rejected	Not Noted	Not Noted	Not Noted	Not Noted
Egg Storage (Prior to use)	Not Noted	Not Noted	Not Noted	Not Noted	Not Noted
Incubation Temperature (^{0}C)	37.5 $\pm$ 0.5	Not Noted	38	Not Noted	38
Relative Humidity (%)	62.5 $\pm$ 7.5	Not Noted	60	Not Noted	60
Egg Rotation?	Yes	Not Noted	Yes	Not Noted	Yes
Checking Egg Viability	Eggs candled on Day 5 and every day thereafter; non-viable embryos removed	Not Noted	Eggs candled on Day 9 and non-viable embryos removed	Not Noted	Eggs candled on Day 9 and non-viable embryos removed
Incubation Period	10 Days	10 Days	9 Days	10 Days	9 Days
Procedure for Opening Egg	Eggshell is scratched around the air cell by a dentist's rotary saw and then pared off. After removal of the inner egg membranes, the CAM is exposed.	Eggshell is scratched around the air cell by a dentist's rotary saw and then pared off. After removal of the inner egg membranes, the CAM is exposed.	Eggshell is scratched around the air cell and opened. After removal of shell, the CAM is exposed.	Not Noted	Eggshell is scratched around the air cell and opened. After removal of shell, the CAM is exposed.
Manipulation of CAM	Not Noted	Not Noted	Not Noted	Not Noted	Not Noted
Investigator Defined Test Substance Classes	Vehicles, antimicrobial agents, oxidation dyes, shampoos, pyrithones, phenols, and isothiazolinones	Substances and formulations	Chemicals from various chemical classes	Surfactants	Chemicals from various chemical classes
Total Test Substances Evaluated	37	190	44	12	5
Test Substance Quantity or Volume	0.2 mL or 0.1 g	0.2-0.3 mL or 0.1 g	0.3 mL	Not Noted	0.3 mL
Test Substance Concentrations Tested	0.1%-100%	Tested concentrations not noted	Various concentrations tested. Solutions of 0.05 to 100% (w/v) tested.	300 mM or 10% mixtures	1% and 10% solution

	Luepke 1985	Luepke and Kemper (1986)	Kalweit et al. (1987)	Reinhardt et al. (1987)	Kalweit et al. (1990)
Application of Solids to CAM	Placed directly on CAM	Placed directly on CAM	All tested substances appear to be solubilized	All tested substances appear to be solubilized	All tested substances appear to be solubilized
Preferred Solvent	Not Noted	Not Noted	Not Noted	Not Noted	Not Noted
Rinse after Test Substance Application?	Yes, 20 seconds after test substance applied rinsed with 5 mL warm water	Yes, 20 seconds after test substance applied rinsed with 5 mL warm water	Not Noted	Not Noted	Not Noted
Evaluation Period	At 0.5, 2, and 5 minutes after test substance applied	At 0.5, 2, and 5 minutes after test substance applied	Up to 300 seconds after test substance applied	Between 0.5 and 5 minutes after test substance applied	Up to 300 seconds after test substance applied
Controls and Test Standards	Vehicle	Not Noted	Not Noted	SDS	Not Noted
Number of Control Eggs	2 eggs	Not Noted	Not Noted	Not Noted	Not Noted
Replicate Eggs	4 eggs	4 eggs	Not Noted	6 eggs	6 eggs per concentration
Number of Replicate Experiments	Not Noted	Not Noted	Not Noted	Not Noted	4
Endpoints Assessed	Hyperemia, hemorrhage, coagulation	Injection, hemorrhage, coagulation	Hemorrhage, vessel lysis, coagulation	Not Noted	Hemorrhage, lysis, coagulation
Endpoint Evaluation	Numerical time-dependent scores for each of the three endpoints. Scoring scheme is noted below.	Numerical time-dependent scores for each of the three endpoints. Scoring scheme is same as Luepke (1985).	The starting second that each of the three endpoints is observed is recorded.	Not Noted	The starting second that each of the three endpoints is observed is recorded.
Analysis Method	Scores are totaled to give a single value (maximum of 21). Mean value of 4 eggs calculated for final value.	Scores are totaled to give a single value (maximum of 21). Mean value of 4 eggs calculated for final value.	Irritation Index is calculated using the formula: (301-sec H)/300*5+(301-sec L)/300*7=(301-sec C)*9; where H=Hemorrhage, L=Lysis; C=Coagulation; sec=starting second	Not Noted	Irritation Index is calculated using the formula: (301-sec H)/300*5+(301-sec L)/300*7=(301-sec C)*9; where H=Hemorrhage, L=Lysis; C=Coagulation; sec=starting second

	Luepke 1985	Luepke and Kemper (1986)	Kalweit et al. (1987)	Reinhardt et al. (1987)	Kalweit et al. (1990)
Classification Scheme	**Non Irritation**: <0.5; **Slight Irritant**: 0.5-3.4; **Moderate Irritant**: 3.5-4.9; **Severe Irritant**: ≥5	**Non Irritation**: <0.5; **Slight Irritant**: 0.5-3.4; **Moderate Irritant**: 3.5-4.9; **Severe Irritant**: ≥5	**Non Irritation**: <0.5; **Slight Irritant**: 0.5-3.4; **Moderate Irritant**: 3.5-4.9; **Severe Irritant**: ≥5	**Non Irritation**: <0.5; **Slight Irritant**: 0.5-3.4; **Moderate Irritant**: 3.5-4.9; **Severe Irritant**: ≥5	**Practically None**: 0-0.9; **Slight Irritation**: 1-4.9; **Moderate Irritation**: 5-8.9; **Strong Irritation**: 9-21
GLP Compliance?	Not Noted	Not Noted	Not Noted	Not Noted	Not Noted
Notes			Cites Luepke (1985) as basis for protocol	Cites Luepke (1985) as basis for protocol and analysis method	

	Lawrence et al. (1990)	Sterzel et al. (1990)	van Erp et al. (1990)	Blein et al. (1991)	CEC (1991)	Hagino et al. (1991)	Gettings et al. (1991)
Hen Strain	Not Noted	Not Noted	Not Noted	White Leghorn	Lohmann Selected Leghorn	White Leghorn	Lohmann's Selected White Leghorn LSL
Egg Criteria for Use	Not Noted	Not Noted	Not Noted	Not Noted	Weight range of eggs between 50 g and 60 g	Not Noted	Not Noted
Egg Storage (Prior to use)	Not Noted	Not Noted	Not Noted	Not Noted	Not Noted	Not Noted	Not Noted
Incubation Temperature (^{0}C)	Not Noted	Not Noted	Not Noted	37.5 $\pm$ 0.5	37.5 $\pm$ 0.5	37.6	Not Noted
Relative Humidity (%)	Not Noted	Not Noted	Not Noted	62.5 $\pm$ 7.5	62.5 $\pm$ 7.5	about 70	Not Noted
Egg Rotation?	Yes	Not Noted	Yes, once each hour	Not Noted	Yes	Yes, once per hour	Not Noted
Checking Egg Viability	Not Noted	Not Noted	Not Noted	Not Noted	Eggs candled on Day 5 and every day thereafter; non-viable embryos removed	Not Noted	Not Noted
Incubation Period	10 Days	9 Days	10 Days	10 Days	10 Days	10 Days	10 Days
Procedure for Opening Egg	Eggshell over the air space was removed with a small rotary cutter on Day 10. Small drop of water placed and spread over the shell membrane to aid in removal of the membrane without damaging the CAM.	Egg shell was scratched above the air chamber with a dentist's rotary saw blade and piece of the shell removed.	Shell with the attached membrane was removed up to the margin of the air chamber. The inner egg membrane was the eliminated to expose the CAM.	Eggshell pierced in the region of the air chamber to expose CAM.	Egg shell was scratched around the air chamber with a rotating dentist saw blade and then pared off. The inner shell was removed and the CAM was layed open.	Eggshell above air space was removed. Drop of water was placed on shell membrane to avoid capillary bleeding. CAM was exposed using forceps.	Not Noted
Manipulation of CAM	Not Noted	Not Noted	Not Noted	Not Noted	Not Noted	Not Noted	Not Noted
Investigator Defined Test Substance Classes	Commercial and industrial products	Fatty acid derivatives	Chemicals from various chemical classes	Chemicals from various chemical classes	Chemicals from various chemical classes	Surfactants (cationic, anionic, non ionic,) and amphoteric agents	Hydro-alcoholic formulations
Total Test Substances Evaluated	34	10	147	40	21	12	10
Test Substance Quantity or Volume	0.2 mL or 0.1 g	0.3 mL	0.2 mL	Not Noted	0.3 mL or 0.1 g	0.2 mL	Not Noted
Test Substance Concentrations Tested	Tested concentrations not noted	Not Noted. Data presented for tested concentrations of 0.5% and 1%	Not Noted	Undiluted or 10% solution	0.1% to 100%	10% solution	Not Noted

	Lawrence et al. (1990)	Sterzel et al. (1990)	van Erp et al. (1990)	Blein et al. (1991)	CEC (1991)	Hagino et al. (1991)	Gettings et al. (1991)
Application of Solids to CAM	Not Noted	All tested substances appear to be solubilized	Not Noted	All tested substances appear to be solubilized	Placed directly on CAM	All tested substances appear to be solubilized	Not Noted
Preferred Solvent	Not Noted	Not Noted	Not Noted	Not Noted	Not Noted	Not Noted	Not Noted
Rinse after Test Substance Application?	20 seconds after application of test substance, rinsed with 5 mL distilled water	Not Noted	Not Noted	Not Noted	Yes, for solids. 20 seconds after application of test substance, rinsed with 5 mL warm water or saline solution	Yes, 20 seconds after test substance applied rinsed with water	Not Noted
Evaluation Period	At 0.5, 2, and 5 minutes after test substance applied	Up to 300 seconds after test substance applied	At 0.5, 2, and 5 minutes after test substance applied	At 0.5, 2, and 5 minutes after test substance applied	Up to 300 seconds after test substance applied	At 0.5, 2, and 5 minutes after test substance applied	Not Noted
Controls and Test Standards	Not Noted	Laureth-8-sulfate	Negative: blank, Reference Substances: toluene and acetone	Yes, but test substances used are not noted	Not Noted	Not Noted	Not Noted
Number of Control Eggs	Not Noted	Not Noted	3 eggs per control or reference substance	2 eggs	Not Noted	Not Noted	Not Noted
Replicate Eggs	6 eggs per test substance	6 eggs	Not Noted	6 eggs	Minimum 6 eggs	4 eggs per sample	Not Noted
Number of Replicate Experiments	Not Noted	Not Noted	Not Noted	Not Noted	Not Noted	Not Noted	Not Noted
Endpoints Assessed	Injection, hemorrhage, coagulation	Blood vessels and albumin examined for such effects as hemorrhage, lysis, and coagulation	Injection, hemorrhage, coagulation	Hyperemia, hemorrhage, coagulation (in terms of opacity and thrombosis)	Hemorrhage, lysis, and coagulation	Hyperemia, hemorrhage, coagulation	Hemorrhage, vascular lysis, coagulation
Endpoint Evaluation	Numerical time-dependent scores for three endpoints.	The starting second that each of the three endpoints is observed is recorded.	Numerical time-dependent scores for three endpoints.	Scored according to Luepke and Kemper (1986) scale	The starting second that each of the three endpoints is observed is recorded.	Numerical time-dependent scores for three endpoints.	The starting second that each of the three endpoints is observed is recorded.
Analysis Method	Scores are totaled to give a single value (maximum of 21). Mean value of 4 eggs calculated for final value.	Reaction Time score is calculated for each egg. Then a ratio of the mean Reaction Time score (for all 6 tested eggs) to the Reaction Time score observed for the reference was calculated.	Scores are totaled to give a single value (maximum of 21). Mean value of 4 eggs calculated for final value.	Not Noted	Irritation Index is calculated using the formula: (301-sec H)/300*5+(301-sec L)/300*7=(301-sec C)*9; where H=Hemorrhage, L=Lysis; C=Coagulation; sec=starting second	Not Noted	Two different analyses were used. Both calculated an irritation index using time (seconds) of appearance of hemorrhage vascular lysis, or coagulation. The differences between the two methods was in the particular calculations used.

	Lawrence et al. (1990)	Sterzel et al. (1990)	van Erp et al. (1990)	Blein et al. (1991)	CEC (1991)	Hagino et al. (1991)	Gettings et al. (1991)
Classification Scheme	**Practically None**: 0-0.9; **Slight Irritation**: 1-4.9; **Moderate Irritation**: 5-8.9; **Strong Irritation**: 9-21	Not Noted	**Non Irritation**: <0.5; **Slight Irritant**: 0.5-3.4; **Moderate Irritant**: 3.5-4.9; **Severe Irritant**: ≥5	Not Noted	Not Noted	Not Noted	Not Noted
GLP Compliance?	Not Noted	Not Noted	Not Noted	Not Noted	Not Noted	Not Noted	Not Noted
Notes	Cites Luepke (1985) as basis for protocol, scoring scheme, and analysis method	Cites Luepke (1985) as basis for protocol	Cites Luepke 1985 as basis for protocol	Cites Luepke and Kemper (1986) as basis for protocol and analysis method		Cites Luepke (1985) as basis for scoring scheme and analysis method	Cites Luepke (1985) as basis for protocol used

	Spielmann et al. (1991)	Bagley et al. (1992)	Rougier et al. (1992)	de Silva et al. (1992)	InVittox Protocol (Spielmann and Liebsh 1992)	Hagino et al. (1993)	Spielmann et al. (1993)
Hen Strain	Not Noted	White Leghorn	White Leghorn	White Leghorn	White Leghorn (Shaver Starcross 288A)	White Leghorn	Not Noted
Egg Criteria for Use	Not Noted	Weight range of eggs between 50 g and 60 g	Weight range of eggs between 50 g and 60 g	Weighing between 50 g and 60 g	Not Noted	Not Noted	Not Noted
Egg Storage (Prior to use)	Not Noted	Not Noted	Not Noted	Not Noted	Not Noted	Not Noted	Not Noted
Incubation Temperature (°C)	Not Noted	37.5 ± 0.5	37.5 ± 0.5	38 ± 0.5	37.5 ± 0.5	37.6	Not Noted
Relative Humidity (%)	Not Noted	62.5 ± 7.5	62.5 ± 7.5	60 ± 5	62.5 ± 7.5	about 70	Not Noted
Egg Rotation?	Not Noted	Yes	Yes	Yes	Yes	Yes, once per hour	Not Noted
Checking Egg Viability	Not Noted	Not Noted	Not Noted	Not Noted	Eggs candled on Day 9 and non-viable embryos removed	Not Noted	Not Noted
Incubation Period	Not Noted	10 Days	10 Days	10 Days	Eggs incubated for 8 days with rotation. After checking for viability on Day 9, viable eggs are placed in incubator with the large end up and incubated without rotation until the next day. Eggs used on Day 10.	10 Days	Not Noted
Procedure for Opening Egg	Not Noted	Egg shell around the air pocket was removed with a dental rotary saw	Egg shell around the air pocket was removed with a dental rotary saw. Inner membrane is removed to expose CAM.	Egg shell around the air pocket was removed using a rotary saw. Inner membrane is removed to expose CAM.	The air cell is marked with a rotating dentist-saw blade and the section is pared off.	Egg shell around the air pocket was removed. Drop of water is placed on the shell membrane to avoid capillary bleeding. Then the CAM is exposed.	Not Noted
Manipulation of CAM	Not Noted	Not Noted	Not Noted	Not Noted	Membrane is moistened with 0.9% NaCl solution at 37. Eggs placed back into the incubator until ready for use (max. of 30 minutes). When ready for use, the NaCl solution is poured off. Then the egg membrane is removed to expose the CAM.	Not Noted	Not Noted
Investigator Defined Test Substance Classes	Chemicals from various chemical classes	Chemicals from various chemical classes, commercial products, and personal care products	Surfactants (non ionic, amphoteric, anionic, cationic) and surfactant-based cosmetic preparations	Surfactants, unidentified chemicals, and cosmetic formulations	Chemicals from various chemical classes	Chemicals from various chemical classes	Chemicals from various chemical classes
Total Test Substances Evaluated	32	32	41	101	27	12	136
Test Substance Quantity or Volume	0.3 mL	0.3 mL or 0.1 g	0.3 mL	0.3 mL	0.3 mL	0.2 mL or 0.2 g	Not Noted
Test Substance Concentrations Tested	1-100% solutions tested	Tested solutions at concentrations that were 10% of those tested *in vivo*	Not Noted	Tested solutions at concentrations that were 10% of those tested *in vivo*	Not Noted	Undiluted or as noted in manuscript	10% solution and threshold concentration (lowest concentration to produce a slight reaction)

	Spielmann et al. (1991)	Bagley et al. (1992)	Rougier et al. (1992)	de Silva et al. (1992)	InVittox Protocol (Spielmann and Liebsh 1992)	Hagino et al. (1993)	Spielmann et al. (1993)
Application of Solids to CAM	All tested substances appear to be solubilized	All tested substances appear to be solubilized	Not Noted	Placed directly on CAM	All tested substances appear to be solubilized	Placed directly on CAM	All tested substances appear to be solubilized
Preferred Solvent	Not Noted	Not Noted	Not Noted	Not Noted	0.9% NaCl or olive oil	Not Noted	Not Noted
Rinse after Test Substance Application?	Not Noted	20 seconds after test substance applied rinsed with 5 mL warm water	Not Noted	For opaque, colored, or solid substances: after 20 seconds test substance rinsed with 5 mL warm saline	Not Noted	Yes, after 20 seconds	Insoluble substances: Yes, after 5 minutes
Evaluation Period	Up to 300 seconds after test substance applied	At 0.5, 2, and 5 minutes after test substance applied	At 0.5, 2, and 5 minutes after test substance applied	At 0.5, 2, and 5 minutes after test substance applied	Up to 300 seconds after test substance applied	At 0.5, 2, and 5 minutes after test substance applied	Not Noted
Controls and Test Standards	Not Noted	Vehicle	Not Noted	Not Noted	1% SDS and 0.1 N NaOH	Not Noted	Not Noted
Number of Control Eggs	Not Noted	2 eggs	Not Noted	Not Noted	2 eggs for each control substance	Not Noted	Not Noted
Replicate Eggs	3 eggs per concentration	4 eggs	4 eggs	4 eggs	3 eggs	4 eggs	3 eggs
Number of Replicate Experiments	2	Not Noted	Not Noted	Not Noted	2	Not Noted	Not Noted
Endpoints Assessed	Hemorrhage, coagulation, lysis	Hyperemia, hemorrhage, coagulation	Hyperemia, hemorrhage, coagulation	Hyperemia, hemorrhage, coagulation (opacity and/or thrombosis)	Hemorrhage, vessel lysis, coagulation	Hyperemia, hemorrhage, coagulation	Not Noted
Endpoint Evaluation	The starting second that each of the endpoints is observed is recorded	Numerical time-dependent scores for three endpoints.	Numerical time-dependent scores for three endpoints.	Numerical time-dependent scores for three endpoints.	The starting second that one of the endpoints is observed is recorded. The degree of severity of each endpoint after the 5 minutes is noted. Severity is ranked from 0 (no reaction) to 3 (severe reaction).	Numerical time-dependent scores for three endpoints.	Not Noted
Analysis Method	Irritation Score calculated. Mean Irritation Score is calculated from individual assays.	Scores are totaled to give a single value (maximum of 21). Mean value of 4 eggs calculated for final value.	Scores are totaled to give a single value (maximum of 21). Mean value of 4 eggs calculated for final value.	Scores are totaled to give a single value (maximum of 21). Mean value of 4 eggs calculated for final value.	Irritation Score (IS; determined at 10% concentration) and Irritation Threshold Concentration (ITC; highest concentration producing a slight reaction during observation period) calculated. Mean IS of 3 eggs calculated for each experiment. Mean value over both experiments calculated for IS and ITC.	Not Noted	Irritation Score (IS; determined at 10% concentration) and Irritation Threshold Concentration (ITC; lowest concentration producing a slight reaction during observation period) calculated

	Spielmann et al. (1991)	Bagley et al. (1992)	Rougier et al. (1992)	de Silva et al. (1992)	InVittox Protocol (Spielmann and Liebsh 1992)	Hagino et al. (1993)	Spielmann et al. (1993)
Classification Scheme	**Practically None**: 0-0.9; **Slight Irritation**: 1-4.9; **Moderate Irritation**: 5-9.9; **Strong Irritation**: 10-21	Not Noted	Not Noted	Not Noted	**Non/Slight:** ITC > 10 and IS <10; Moderate: (1) ITC between 2.5 and 10 and IS <16 and weak or no reaction noted or (2) ITC > 10 and IS > 16 or (3) ITC > 10 and IS < 16 and severe reaction is noted; **Irritant:** (1) ITC between 1 and 2.5 and IS < 16 or (2) ITC between 2.5 and 10 and IS >16 or (3) ITC between 2.5 and 10 and IS >16 and severe reaction noted after 5 minutes; **Severe:** (1) ITC < 1% or (2) ITC between 1 and 2.5% and IS > 16 or (3) ITC between 2.5 and 10 and IS < 16 and severe reaction noted after 1 minute	Not Noted	**Non/Slight:** ITC > 10 and IS <16; **Moderate:** ITC <10 and IS <16 or ITC >10 and IS 16; **Irritant:** ITC <2.5 and IS <16 or ITC <10 and IS >16; **Severe**: ITC < 1% or ITC <2.5% and IS > 16
GLP Compliance?	Not Noted	Not Noted	Not Noted	Not Noted	Not Noted	Not Noted	Not Noted
Notes	Cites Luepke (1985) as modified by Kalweit (1990) as basis for protocol and analysis method	Cites Luepke and Kemper (1986) as basis for protocol	Cites Luepke (1985) as basis for scoring scheme	Cites Luepke (1981) as basis for protocol and scoring scheme	Not Noted	Cites Luepke (1985) as basis for scoring scheme	Cites Luepke (1985) as modified by Kalweit (1990) and Spielmann (1991) as basis for protocol

	Gettings et al. (1994)	Vinardell and Macián (1994)	Balls et al. (1995) (Non-Transparent Substances)	Balls et al. (1995) (Transparent Substances)	Kojima et al. (1995)
Hen Strain	White Leghorn	Leghorn SA31	Not Noted	Not Noted	Not Noted
Egg Criteria for Use	Not Noted	Not Noted	Not Noted	Not Noted	Not Noted
Egg Storage (Prior to use)	Not Noted	Not Noted	Not Noted	Not Noted	Not Noted
Incubation Temperature (^{0}C)	38	Not Noted	Not Noted	Not Noted	37.6
Relative Humidity (%)	60	Not Noted	Not Noted	Not Noted	about 70
Egg Rotation?	Yes	Not Noted	Not Noted	Not Noted	Yes, once per hour
Checking Egg Viability	Candled on Day 9 and non-viable embryos removed	Not Noted	Not Noted	Not Noted	Not Noted
Incubation Period	9 Days	10 Days	9 Days	9 Days	10 Days
Procedure for Opening Egg	Eggshell was scratched around the air cells and a small aperture was opened.	Egg shell was scratched around the air cell by a dentist's rotary saw and then pared off. After removal of the inner membrane, the CAM was exposed.	Not Noted	Not Noted	A portion of the egg shell was removed and a drop of water was placed onto the shell membrane to avoid bleeding. Then the CAM was exposed with forceps.
Manipulation of CAM	Not Noted	Not Noted	Not Noted	Not Noted	Not Noted
Investigator Defined Test Substance Classes	Oil/water -based personal care formulations	Commercial disinfectants	Chemicals selected from the ECETOC database (acids, acyl halides, alcohols, aldehye, alkalis, esters, hetercyclics, hydrocarbons, inorganic chemicals, ketones, organophosphates, pesticides, surfactants, misc.)	Chemicals selected from the ECETOC database (acids, acyl halides, alcohols, aldehye, alkalis, esters, hetercyclics, hydrocarbons, inorganic chemicals, ketones, organophosphates, pesticides, surfactants, misc.)	Surfactants, solvents, formaldehyde
Total Test Substances Evaluated	18	6	59	59	24
Test Substance Quantity or Volume	0.3 mL	0.3 mL	Not Noted	Not Noted	0.2 mL
Test Substance Concentrations Tested	3 concentrations tested: threshold concentration, 10% solution, undiluted	Diluted or undiluted	Not Noted	Not Noted	10% solution

	Gettings et al. (1994)	Vinardell and Macián (1994)	Balls et al. (1995) (Non-Transparent Substances)	Balls et al. (1995) (Transparent Substances)	Kojima et al. (1995)
Application of Solids to CAM	All tested substances appear to be solubilized	All tested substances appear to be solubilized	Not Noted	Not Noted	All tested substances appear to be solubilized
Preferred Solvent	Not Noted	Distilled water	Not Noted	Not Noted	Not Noted
Rinse after Test Substance Application?	Not Noted	Not Noted	After 3 minute exposure	Not Noted	Yes, after 20 seconds test substance was rinsed with water
Evaluation Period	Up to 300 seconds after test substance applied	Up to 300 seconds after test substance applied	Within 30 seconds of rinsing	Up to 300 seconds after test substance applied	At 0.5, 2, and 5 minutes after test substance rinsed
Controls and Test Standards	Not Noted	0.1 M NaOH, 1% SDS, 0.9% NaCl, distilled water	5% Texapon AV (internal reference standard)	5% Texapon AV (internal reference standard)	Not Noted
Number of Control Eggs	Not Noted	2 eggs per substance	Not Noted	Not Noted	Not Noted
Replicate Eggs	3 eggs per concentration tested	6 eggs	6 eggs	6 eggs	4 eggs
Number of Replicate Experiments	2	Not Noted	Not Noted	Not Noted	Not Noted
Endpoints Assessed	Hemorrhage, coagulation, lysis	Hemorrhage, vasoconstriction, coagulation	Hemorrhage, lysis, coagulation	Hemorrhage, lysis, coagulation	Hyperemia, hemorrhage, coagulation
Endpoint Evaluation	Seconds for the three endpoints recorded (see Kalweit, 1990)	The starting second that each of the endpoints is observed is recorded	Endpoints scored from 0 (no reaction) to 3 (strong reaction)	The starting second that each of the endpoints is observed is recorded	Not Noted
Analysis Method	Calculation of an Irritation Score for each egg. Mean value of individual Irritation Scores calculated.	Irritancy Potential calculated using the formula: (301-sec H)/300*5+(301-sec v)/300*7=(301-sec C)*9; where H=Hemorrhage, V=vasoconstriction; C=Coagulation of protein or blood; sec=starting second	Calculation of "S Score". "S Score" is calculated using the most sensitive endpoint (endpoint can change from chemical to chemical). The scores recorded for the most sensitive endpoint are summarized for the 6 eggs. (Maximum for 6 eggs is 18)	Computer program calculates an Irritation Index. Irritation Index Is used to calculate a "Q Score". "Q Score" is a comparison of the Irritation Index of a test chemical with that of the reference chemical. If the effect of test chemical is identical to reference, Q is 1.0. If effect of test chemical is less irritating, Q is lower. If effect of test chemical is more irritating, Q is higher.	Score was calculated based on the time of onset for each endpoint. Mean value of 4 eggs calculated.

	Gettings et al. (1994)	Vinardell and Macián (1994)	Balls et al. (1995) (Non-Transparent Substances)	Balls et al. (1995) (Transparent Substances)	Kojima et al. (1995)
Classification Scheme	**Practically None**: 0-0.9; **Slight Irritation**: 1-4.9; **Moderate Irritation**: 5-9.9; **Strong Irritation**: 10-21	**Practically None**: 0-0.9; **Slight Irritation**: 1-4.9; **Moderate Irritation**: 5-8.9; **Strong Irritation**: 9 21	**Non Irritation**: S < 6 ; **Moderately Irritating**: 6<= S <15; **Severely Irritating**: S ≥15	**Non Irritating**: Q < 1.5; **Moderately Irritating:** 1.5 < Q <2.0; **Severely Irritating**: Q < 2.0	Not Noted
GLP Compliance?	Not Noted	Not Noted	Yes	Yes	Not Noted
Notes	Cites Luepke (1985) as basis for scoring scheme	Cites Ergatt/Frame Data Bank (1990) as basis for protocol and analysis method			Cites Luepke (1985) and Luepke and Wallat (1985) as basis for scoring scheme and analysis method.
	To study the effects of slow acting materials, CAM scored 15 and 30 minutes after application on range from 0 (no reactions) to 3 (strong reactions)				

	Spielmann (1995)	Macian et al. (1996)	Gilleron et al. (1996)	Gettings et al. (1996) (HET-CAM I, II)	Gettings et al. (1996) (HET-CAM III)
Hen Strain	White Leghorn (Shaver Starcross 288A)	Leghorn SA31	White Essex	Lohmann's Selected White Leghorn	White Leghorn
Egg Criteria for Use	Weighing 50-60 g. Eggs are candled and non-viable eggs removed	Not Noted	Eggs were 7 days old prior to start of incubation and weighed 60 $\pm$ 5 g	Not Noted	Not Noted
Egg Storage (Prior to use)	Not Noted	Not Noted	Not Noted	Not Noted	Not Noted
Incubation Temperature (^{0}C)	37.5 $\pm$ 0.5	Not Noted	37.0 $\pm$ 0.5	Not Noted	38 $\pm$ 0.5
Relative Humidity (%)	62.5 $\pm$ 7.5	Not Noted	62.5 $\pm$ 1.5	Not Noted	60 $\pm$ 5
Egg Rotation?	Yes, for 8 days	Not Noted	Yes, with large ends upward for 9 days	Not Noted	Yes
Checking Egg Viability	Eggs candled on Day 9 and non-viable eggs removed. Eggs replaced in incubator with large ends up but were not rotated	Not Noted	Not Noted	Not Noted	Candled on Day 9 and returned to incubator in vertical position
Incubation Period	10 Days	10 Days	10 Days	10 Days	10 Days
Procedure for Opening Egg	Air cell is marked using a rotating dentist-saw blade. The egg shell is then pared off.	Egg shell was scratched around the air cell by a dentist's rotary saw and pared off. After removal of inner egg membrane, the CAM was exposed.	Eggs were candled and non-viable eggs were discarded. The airspace delimited by the inner membrane at the large end of the egg was marked. The eggshell was removed using a dentist's rotating saw blade. The inner membrane was moistened with 1.5-2.0 mL of 0.9% NaCl and the egg was returned to the incubator (at 37) for a maximum of 20 mins. After incubation, the NaCl solution was removed, using a vacuum pump, and the inner membrane was removed with forceps.	Not Noted	Shell and inner shell membrane were removed around the area defined by the air cell
Manipulation of CAM	Membrane is moistened with 0.9% NaCl solution at 37. Eggs are then placed back in to the incubator until ready for use (maximum of 30 minutes). When ready for use the NaCl solution is poured off and the membrane is removed with tapered forceps.	Not Noted	A test substance applicator (TSA), which is comprised of a perlon mesh (pore diameter = 63 micron) locked between two Teflon rings, was placed on the CAM	Not Noted	Not Noted
Investigator Defined Test Substance Classes	Not Applicable	Polyoxyethylene non ionic surfactants	Chemicals from various chemical classes	Surfactant-based personal care formulations	Surfactant-based personal care formulations
Total Test Substances Evaluated	Not Applicable	9	46	25	25
Test Substance Quantity or Volume	0.3 mL (for insoluble solids: put some grains onto CAM to cover approximately half the surface)	0.3 mL	0.3 mL or 0.3 g of test substance placed inside the TSA	0.3 mL	0.1 mL
Test Substance Concentrations Tested	10% solution and several additional concentrations as determined by investigator	Different concentrations	Undiluted	3 concentrations tested: threshold concentration, 10% solution, undiluted	10% solution

	Spielmann (1995)	Macian et al. (1996)	Gilleron et al. (1996)	Gettings et al. (1996) (HET-CAM I, II)	Gettings et al. (1996) (HET-CAM III)
Application of Solids to CAM	All tested substances appear to be solubilized	All tested substances appear to be solubilized	Placed inside TSA	All test substances appear to be in liquid form	All test substances appear to be in liquid form
Preferred Solvent	0.9% NaCl or olive oil	Soluble Substances: 0.9% NaCl; Insoluble Substances: carboxymethylcellulose	0.9% NaCl	Not Noted	Not Noted
Rinse after Test Substance Application?	For insoluble test chemical: after 5 minutes rinse off with NaCl	Not Noted	TSA (which contains the test substance) is removed after 20 seconds	Not Noted	After 20 seconds, with a saline rinse
Evaluation Period	Up to 300 seconds after test substance applied	Up to 300 seconds after test substance applied	Up to 300 seconds after test substance applied	Up to 300 seconds after test substance applied	At 0.5, 2, and 5 minutes after test substance rinsed
Controls and Test Standards	1% SDS and 0.1 N NaOH	1% SDS and 0.1 N NaOH	Positive Controls: benzalkonium chloride, dimethylformamide, imidazole	Not Noted	Not Noted
Number of Control Eggs	2 eggs per standard	2 eggs per standard	Not Noted	Not Noted	Not Noted
Replicate Eggs	3 eggs	6 eggs	3	3 eggs per concentration tested	Not Noted
Number of Replicate Experiments	2	Not Noted	3	Not Noted	Not Noted
Endpoints Assessed	Hemorrhage, vascular lysis, coagulation	Hemorrhage, vasoconstriction, coagulation	Hemorrhage, lysis, coagulation	Hemorrhage, lysis, coagulation	Dilation, hemorrhage, coagulation
Endpoint Evaluation	The starting second that each of the endpoints is observed is recorded. Additionally, the severity of the reaction is graded (between 0 and 3) after 5 minutes of exposure to test substance.	The starting second that each of the endpoints is observed is recorded	The starting second that each of the endpoints is observed is recorded	The starting second that each of the endpoints is observed is recorded (see Kalweit 1990)	Numerical time-dependent scores for three endpoints.
Analysis Method	Irritation Score (IS; determined at 10% concentration) and Irritation Threshold Concentration (ITC; lowest concentration producing a slight reaction during observation period) calculated. Mean IS of 3 eggs calculated for each experiment. Mean value over both experiments calculated for IS and ITC.	Irritancy Potential calculated using the formula: (301-sec H)/300*5+(301-sec v)/300*7=(301-sec C)*9; where H=Hemorrhage, V=vasoconstriction; C=Coagulation of protein or blood; sec=starting second. Mean value and SEM of 6 separate experiments are calculated.	Irritation Index calculated using the formula: (301-sec H)/300*5+(301-sec L)/300*7=(301-sec C)*9; where H=Hemorrhage, L=Vessel Lysis; C=Coagulation; sec=starting second. Mean of 3 assays were calculated. Reproducibility also was assessed.	Mean Irritation Score (IS; determined at 10% concentration) of 3 eggs is calculated. The Irritation Threshold Concentration (ITC; lowest concentration producing a slight reaction during observation period) is calculated.	Time-dependent scores were used to calculate a single value (maximum of 21).

	Spielmann (1995)	Macian et al. (1996)	Gilleron et al. (1996)	Gettings et al. (1996) (HET-CAM I, II)	Gettings et al. (1996) (HET-CAM III)
Classification Scheme	**Non/Slight: I**TC > 10 and IS <10; Moderate: (1) ITC between 2.5 and 10 and IS <16 and weak or no reaction noted or (2) ITC > 10 and IS > 16 or (3) ITC > 10 and IS < 16 and severe reaction is noted; **Irritant:** (1) ITC between 1 and 2.5 and IS < 16 or (2) ITC between 2.5 and 10 and IS >16 or (3) ITC between 2.5 and 10 and IS >16 and severe reaction noted after 5 minutes; **Severe:** (1) ITC < 1% or (2) ITC between 1 and 2.5% and IS > 16 or (3) ITC between 2.5 and 10 and IS < 16 and severe reaction noted after 1 minute	**Practically None**: 0-0.9; **Slight Irritation**: 1-4.9; **Moderate Irritation**: 5-8.9; **Strong Irritation**: 9-21	**Non Irritant**: 0-4.9; **Irritant**: 5.0-21	**Irritant (According to FHSA)**: IS ≥ 5.1 or IS/ITC ≥ 3.0	**Irritant (According to FHSA)**: Score ≥ 4.83
GLP Compliance?	Not Noted	Not Noted	Not Noted	Not Noted	Not Noted
Notes					

	Spielmann et al. (1996)	Brantom et al. (1997)	Budai et al. (1997)	Gilleron et al. (1997)	Spielmann et al. (1997) (HETCAM-1)	Spielmann et al. (1997) (HETCAM-1, Laboratory A)
Hen Strain	Not Noted	Not Noted	White Leghorn (Shaver Starcross 288)	White Essex	White Leghorn	White Leghorn
Egg Criteria for Use	Not Noted	Not Noted	Eggs were candled and non viable eggs were discarded	Eggs were 7 days old	Not Noted	Not Noted
Egg Storage (Prior to use)	Not Noted	Not Noted	Not Noted	Not Noted	Not Noted	Not Noted
Incubation Temperature (^{o}C)	Not Noted	Not Noted	37	37 $\pm$ 0.5	38	38
Relative Humidity (%)	Not Noted	Not Noted	60-70	52.5 $\pm$ 2.5	60	60
Egg Rotation?	Not Noted	Not Noted	Yes, for 8 days	Not Noted	Yes	Yes
Checking Egg Viability	Not Noted	Not Noted	Eggs were candled on the 9th day and non viable eggs were discarded.	Not Noted	Not Noted	Not Noted
Incubation Period	Not Noted	9 Days	9 Days, then eggs were candled and non-viable eggs were discarded. Eggs were placed in incubator (but not rotated) until Day 10	10 Days	9 Days	9 Days
Procedure for Opening Egg	Not Noted	Not Noted	The air cell of eggs were marked and the section of shell was removed with scissors. The membrane was moistened with 0.9% NaCl and eggs were placed in incubator until ready for use.	Not Noted	Egg shells were opened at the air cell	Egg shells were opened at the air cell
Manipulation of CAM	Not Noted	Not Noted	Not Noted	A test substance applicator (TSA) was placed on the CAM	Not Noted	Not Noted
Investigator Defined Test Substance Classes	Chemicals from various chemical classes	Substances representative of cosmetic ingredients and finished personal product formulations	Pesticides	Chemicals from various chemical classes	Inorganic chemicals, aliphatic organics, aromatics, heterocyclics	See Gettings et al. lists
Total Test Substances Evaluated	200	55	4	60	133	53
Test Substance Quantity or Volume	Not Noted	Not Noted	0.3 mL	0.3 mL or 0.3 g of test substance placed inside the TSA	0.3 mL	0.3 mL
Test Substance Concentrations Tested	1-100% solutions tested	Not Noted	1, 10, and 100%	Not Noted	Undiluted and 10% concentration	Undiluted and 10% concentration

	Spielmann et al. (1996)	Brantom et al. (1997)	Budai et al. (1997)	Gilleron et al. (1997)	Spielmann et al. (1997) (HETCAM-1)	Spielmann et al. (1997) (HETCAM-1, Laboratory A)
Application of Solids to CAM	All tested substances appear to be solubilized	All test substances appear to be in liquid form	All tested substances appear to be solubilized	Placed inside TSA	All tested substances appear to be solubilized	Not Noted
Preferred Solvent	0.9% NaCl or olive oil	Not Noted	Not Noted	Not Noted	0.9% NaCl or olive oil	Not Noted
Rinse after Test Substance Application?	Yes, after 5 minutes (for substances that were insoluble in eater or oil and were colored).	Non-trasnparent substances were rinsed off of the CAM after 30 seconds	Not Noted	TSA (which contains the test substance) is removed after 20 seconds	Not Noted	Not Noted
Evaluation Period	Up to 300 seconds after test substance applied	Up to 300 seconds for transparent test susbtances	Up to 300 seconds after test substance applied	Up to 300 seconds after test substance applied	Up to 300 seconds after test substance applied	Up to 300 seconds after test substance applied
Controls and Test Standards	Not Noted	5% solution of Texapon ASV	Standards: 1% SDS and 0.1M NaOH; Control: 0.9% NaCl	Not Noted	0.1 N NaOH, 0.1% SDS, 0.9% NaCl	0.1 N NaOH, 0.1% SDS, 0.9% NaCl
Number of Control Eggs	Not Noted	Not Noted	2 eggs per control and standard	Not Noted	Not Noted	Not Noted
Replicate Eggs	Not Noted	6 eggs	6 eggs for each concentration	Not Noted	3 eggs	3 eggs
Number of Replicate Experiments	Not Noted	Not Noted	4	Not Noted	Not Noted	Not Noted
Endpoints Assessed	Hemorrhage, coagulation, lysis	Hemorrhage, coagulation, lysis	Hemorrhage, vascular lysis, coagulation	Hemorrhage, lysis, coagulation	Hemorrhage, lysis, coagulation	Hemorrhage, lysis, coagulation
Endpoint Evaluation	The starting second that each of the endpoints is observed is recorded	The starting second that each of the endpoints is observed is recorded for transparent substances and quality of the effects is determined for the nontransparent substances	The starting second that each of the endpoints is observed is recorded	The starting second that each of the endpoints is observed is recorded	The starting second that each of the endpoints is observed is recorded	Not Noted
Analysis Method	Irritation Score calculated ((301-sec H)/300*5+(301-sec L)/300*7=(301-sec C)*9; where H=Hemorrhage, L=Lysis; C=Coagulation; sec=starting second) for 10% solution calculated (IS10). Irritation Threshold (ITC; lowest concentration of a test substance to induce an irritant reaction on the CAM) calculated.	Computer program calculates an Irritation Index. Irritation Index Is used to calculate a "Q Score". "Q Score" is a comparison of the Irritation Index of a test chemical with that of the reference chemical. If the effect of test chemical is identical to reference, Q is 1.0. If effect of test chemical is less irritating, Q is lower. If effect of test chemical is more irritating, Q is higher. Calculation of "S Score". "S Score" is calculated using the most sensitive endpoint (endpoint can change from chemical to chemical). The scores recorded for the most sensitive endpoint are summarized for the 6 eggs. (Maximum for 6 eggs is 18)	Not Noted	Irritation Index calculated using the formula: (301-sec H)/300*5+(301-sec L)/300*7=(301-sec C)*9; where H=Hemorrhage, L=Vessel Lysis; C=Coagulation; sec=starting second. Mean value and SEM of are calculated.	Irritation Score calculated using the formula: (301-sec H)/300*5+(301-sec L)/300*7=(301-sec C)*9; where H=Hemorrhage, L=Lysis; C=Coagulation; sec=starting second.	Not Noted

	Spielmann et al. (1996)	Brantom et al. (1997)	Budai et al. (1997)	Gilleron et al. (1997)	Spielmann et al. (1997) (HETCAM-1)	Spielmann et al. (1997) (HETCAM-1, Laboratory A)
Classification Scheme	**BGA Classification Model: Non/Slight**: ITC > 10 and IS10 <16; **Moderate**: (1) ITC > 16 and IS10 > 16 or (2) ITC < 10 and IS10 < 16; **Irritant (R36)**: (1) ITC < 10 and IS10 > 16 or (2) ITC < 2.5 and IS10 < 16; **Severe (R41)**: (1) ITC ≤ 1% or (2) ITC between 1 and 2.5% and IS10 ≥ 16. **Proposed model using mtc10 (see notes below): R41**: mtc10 < 50 seconds.	**Q-SCORE: Slightly Irritating:** Q <= 0.8; **Moderately Irritating:** 0.8 < Q <1.2; **Irritating:** 1.2 <=Q<2.0; **Severely Irritating:** Q <= 2.0; **S-SCORE: Slightly Irritating** S < 6 ; **Moderately Irritating**: 6<= S <12; **Irritating**: 12 <= S ≥16; **Severely Irritating** S>=16	**No Irritation**: 0-0.9; **Weak Irritation**: 1-4.9; **Moderate Irritation**: 5-8.9; **Severe Irritation**: 9-21	**Non Irritant**: 0-4.9; **Irritant**: 5.0-21	Not Noted	Not Noted
GLP Compliance?	Yes	Yes	Not Noted	Not Noted	Not Noted	Not Noted
Notes	Cites Luepke (1985) as basis for protocol. Cites Kalweit (1990) and Spielmann (1991) as publishing the protocol used. Cites the standard protocol for the test method as InVittox Protocol.			Cites Gilleron 1996 as basis for protocol and analysis method		
	Nine additional endpoints were conducted using the raw data and the IS and IT scores in this analysis. Of these the endpoints evaluated, that best correlated with in vivo classification was mtc10 (mean detection time for appearance of coagulation when using a 10% solution).					

	Spielmann et al. (1997) (HETCAM-II)	Spielmann et al. (1997) (HETCAM-III)	Vives et al. (1997)	Doucet et al. (1999)	Hagino et al. (1999)	Lönnroth et al. (1999)
Hen Strain	White Leghorn	White Leghorn	Leghorn SA31	White Leghorn	White Leghorn	Not Noted
Egg Criteria for Use	Not Noted	Not Noted	Not Noted	Eggs weighed between 50-60 g	Not Noted	Not Noted
Egg Storage (Prior to use)	Not Noted	Not Noted	Not Noted	Not Noted	Not Noted	Not Noted
Incubation Temperature (^{0}C)	37.8	37.5 $\pm$ 1	Not Noted	37.5 $\pm$ 1	37.6	38
Relative Humidity (%)	55%	40-60	Not Noted	60 $\pm$ 5	about 70	Not Noted
Egg Rotation?	Not Noted	Not Noted	Not Noted	Yes, eggs were placed on their long axis	Yes, once an hour	Not Noted
Checking Egg Viability	Not Noted	Not Noted	Not Noted	Not noted	Not Noted	Not Noted
Incubation Period	9 Days	10 Days	10 Days	10 Days	10 Days	9 Days
Procedure for Opening Egg	Not Noted	Not Noted	Egg shell was scratched around the air cell by a dentist's rotary saw and pared off. After removal of inner egg membrane, the CAM was exposed.	Eggshell around the airspace was removed. 5 mL of 0.9% NaCl was placed on the inner membrane and then the CAM was exposed	Portion of the egg shell above the air space was removed.	Egg shell above the air cell was opened using a dental drill saw blade and forceps. The shell membrane was moistened with 0.9% NaCl solution at 37 degrees Celsius. The NaCl was aspirated, the shell membrane removed, and CAM exposed.
Manipulation of CAM	Not Noted	Not Noted	Not Noted	Not noted	Drop of water is placed on the membrane (to avoid capillary bleeding). A silicone rubber ring is placed on the CAM.	Eggs were examined using a microscope. Photo-micrographs were taken 2 minutes after application of each material.
Investigator Defined Test Substance Classes	Surfactants and formulations	Inorganics, aliphatics, alicyclics, heterocyclic, alkyl derivatives, polymers	Surfactants derived from lysine	Skin-care products, sunscreen products, surfactant-based products, and alcoholic products	Surfactant, polyols, color additives, organic salts, PABA derivative, esters, inorganic chemicals, alcohols, amines, alkanolamines, carboxylic acids	Dental polymer products
Total Test Substances Evaluated	42	97	6	40	39	8
Test Substance Quantity or Volume	0.3 mL	0.3 mL	0.3 mL	0.3 mL	0.2 mL (placed inside the rubber ring on the CAM). Solids (0.2 g) are reduced to a fine powder with a No. 200 sieve and placed inside a rubber ring on the CAM.	0.1-0.2 mL
Test Substance Concentrations Tested	Not Noted	Solids and liquids were generally tested undiluted; turbid materials were diluted to concentrations that allowed observation of the CAM	Different concentrations	Surfactant products: 3% concentration; Alcoholic products: 10% alcohol concentration; Emulsion type products: undiluted	0.1, 1, 10, and 100%	Not Noted

	Spielmann et al. (1997) (HETCAM-II)	Spielmann et al. (1997) (HETCAM-III)	Vives et al. (1997)	Doucet et al. (1999)	Hagino et al. (1999)	Lönnroth et al. (1999)
Application of Solids to CAM	All tested substances appear to be solubilized	Not Noted	All tested substances appear to be solubilized	Not Noted	Placed inside rubber ring	All tested substances appear to be solubilized
Preferred Solvent	Not Noted	Water or solvents	Distilled water	Sterilized water	Not Noted	Cell culture media (MEM, 2 mM L-glutamine, 100 IU/mL penicillin, 100 mg/ml Streptomycin, 5% FBS). Solutions were incubated for 24 hrs in a water bath at 37 degrees. The suspensions were centrifuged and the supernatants applied
Rinse after Test Substance Application?	Not Noted	Not Noted	Not Noted	Yes, 20 seconds after test substance application it was rinsed with 5 mL of warm saline by irrigation of the tilted egg	Yes, after 20 seconds with distilled water	Not Noted
Evaluation Period	Up to 300 seconds after test substance applied	At 0.5, 2, and 5 minutes after test substance applied	Up to 300 seconds after test substance applied	At 0.5, 2, and 5 minutes after test substance applied	At 0.5, 2, and 5 minutes after test substance applied	Up to 240 seconds after test substance applied
Controls and Test Standards	Water, other additional controls depend upon the class of chemicals being evaluated	Not Noted	1% SDS and 0.1 N NaOH	Positive control: 0.3% SDS; Negative control: saline solution	Not Noted	Positive: 0.1N NaOH; Negative: 0.9% NaCl
Number of Control Eggs	1 egg per control	Not Noted	2 eggs per standard	2 eggs per control	Not Noted	Not Noted
Replicate Eggs	4 eggs	6 eggs	6 eggs	4 eggs for each test sample	4 eggs	3 eggs
Number of Replicate Experiments	1 assay with 4 eggs or 2 assays with 2 eggs	Not Noted	Not Noted	1	Not Noted	2
Endpoints Assessed	Hyperemia, hemorrhage, coagulation	Hemorrhage, lysis, coagulation	Hemorrhage, vasoconstriction, coagulation	Hyperemia, hemorrhage, coagulation	Hyperemia, hemorrhage, coagulation	Hemorrhage, lysis, coagulation
Endpoint Evaluation	Numerical time-dependent scores for three endpoints.	The starting second that each endpoint is observed is recorded. Additionally, the endpoint score "S" is determined at 0.5, 1, 3, or 5 mins.	The starting second that each of the endpoints is observed is recorded	Numerical time-dependent scores for three endpoints.	Numerical time-dependent scores for three endpoints.	The starting second that each of the endpoints is observed is recorded
Analysis Method	Time-dependent scores were used to calculate a single value (maximum of 21).	"S Score" is calculated using scores recorded for the most sensitive endpoint are summarized for the 6 eggs. (Maximum for 6 eggs is 18); "Q Score" is a comparison of the Irritation Score of a test chemical with that of the reference chemical.	Irritancy Potential calculated using the formula: (301-sec H)/300*5+(301-sec v)/300*7=(301-sec C)*9; where H=Hemorrhage, V=vasoconstriction; C=Coagulation of protein or blood; sec=starting second. Mean value and SEM of 6 separate experiments are calculated.	Mean of the calculated scores were determined for each test substance.	Not Noted	Irritation Score calculated using the formula: (301-sec H)/300*5+(301-sec L)/300*7=(301-sec C)*9; where H=Hemorrhage, L=Lysis; C=Coagulation; sec=starting second

	Spielmann et al. (1997) (HETCAM-II)	Spielmann et al. (1997) (HETCAM-III)	Vives et al. (1997)	Doucet et al. (1999)	Hagino et al. (1999)	Lönnroth et al. (1999)
Classification Scheme	Not Noted	Not Noted	**Practically None**: 0-0.9; **Slight Irritation**: 1-4.9; **Moderate Irritation**: 5-8.9; **Strong Irritation**: 9-21	Not noted	Not Noted	**Practically None**: 0-0.9; **Slight Irritation**: 1-4.9; **Moderate Irritation**: 5-8.9; **Strong Irritation**: 9-21
GLP Compliance?	Not Noted	Not Noted	Not Noted	Not noted	Not Noted	Not Noted
Notes			Cites Ergatt/Frame Data Bank (1990) as basis for protocol	Cites Luepke (1985) as basis for protocol, scoring scheme, and analysis method	Cites Luepke 1985 as basis for scoring method	Cites Kalweit (1990) as basis of protocol

	Schlage et al. (1999)	Steiling et al. (1999)	Budai and Várnagy (2000)	Djabari et al. (2002)
Hen Strain	White Leghorn	White Leghorn (Shaver Starcross 288)	White Leghorn Shaver 288	White Leghorn
Egg Criteria for Use	Not Noted	Less than 1 week after laying (approximate weight about 50 g)	Eggs were candled and all defective ones were discarded	Eggs weighing between 50-65 g
Egg Storage (Prior to use)	Not Noted	Not Noted	Not Noted	Not Noted
Incubation Temperature (^{0}C)	37.5	37.5 $\pm$ 0.5	37	37.8
Relative Humidity (%)	Not Noted	55 $\pm$ 7	60-70	Not Noted
Egg Rotation?	Not Noted	Yes	Yes for first 8 days	Not Noted
Checking Egg Viability	Not Noted	On day 10 the eggs are candled and non-viable eggs removed	On day 9 the eggs are candled and non-viable eggs removed	Not Noted
Incubation Period	9 or 10 Days	10 Days	9 Days, then eggs were candled and non-viable eggs were discarded. Eggs were placed in incubator (but not rotated) until Day 10	10 Days
Procedure for Opening Egg	Egg shell was opened at the airspace with a dentist's saw and the apical parts of the shell were removed.	Egg shell was opened with an electric drill and the white egg membrane was removed	Airspace is marked and section of shell removed with scissors.	Not Noted
Manipulation of CAM	Not Noted	Not Noted	Membrane is moistened with 0.9% NaCl after CAM is exposed. Egg is placed back in incubator until ready for use.	Not Noted
Investigator Defined Test Substance Classes	Cigarette smoke	Substances representing a range of chemicals used in the cosmetics industry	Pesticides	Substances with vegetal, marine, biotechnological, or chemical synthetic origin
Total Test Substances Evaluated	2	100	6	20
Test Substance Quantity or Volume	0.2 mL	0.3 mL	0.3 mL	Not Noted
Test Substance Concentrations Tested	Not Noted	Undiluted	Not Noted	Undiluted and 10%

	Schlage et al. (1999)	Steiling et al. (1999)	Budai and Várnagy (2000)	Djabari et al. (2002)
Application of Solids to CAM	Not Applicable	Amount sufficient to cover at least 25% of the CAM was applied	All tested substances appear to be solubilized	All tested substances appear to be solubilized
Preferred Solvent	Not Applicable	Not Noted	Not Noted	Not Noted
Rinse after Test Substance Application?	Not Noted	For non-transparent substances, rinsed off after 30 seconds	Not Noted	Not Noted
Evaluation Period	Up to 300 seconds after test substance applied	Up to 300 seconds for transparent substances and	Up to 300 seconds after test substance applied	Up to 300 seconds after test substance applied
Controls and Test Standards	PBS	Texapon ASV	0.9% NaCl; solution of 1% SDS and 0.1 M NaOH	Not Noted
Number of Control Eggs	Not Noted	6 eggs	2 eggs for each substance	Not Noted
Replicate Eggs	3 to 4 eggs for each dose	6 eggs	6 eggs	Not Noted
Number of Replicate Experiments	Not Noted	Not Noted	4	Not Noted
Endpoints Assessed	Injection, hemorrhage, coagulation (IHC) or hemorrhage, vessel lysis, coagulation (HVC)	Hemorrhage, lysis, coagulation	Hemorrhage, vessel lysis, coagulation	Hyperemia, hemorrhage, coagulation
Endpoint Evaluation	Numerical time-dependent scores for three endpoints and the starting second that each of the endpoints is observed is recorded.	The starting second that each of the endpoints is observed is recorded for transparent substances and quality of the effects is determined for the nontransparent substances	The starting second that each of the endpoints is observed is recorded	Numerical time-dependent scores for three endpoints.
Analysis Method	A cumulative discontinuous irritation score (maximum value of 21) calculated for IHC and HVC. Additionally, a continuous Irritation Score calculated using the formulation: (301-sec I)/300*5+(301-sec H)/300*7=(301-sec C)*9; where I=Injection, H=Hemorrhage, C=Coagulation, sec=starting second or (301 sec H)/300*5+(301-sec V)/300*7=(301-sec C)*9; where H=Hemorrhage, L=Vessel Lysis, C=Coagulation, sec=starting second	Computer program calculates an Irritation Index. Irritation Index Is used to calculate a "Q Score". "Q Score" is a comparison of the Irritation Index of a test chemical with that of the reference chemical. If the effect of test chemical is identical to reference, Q is 1.0. If effect of test chemical is less irritating, Q is lower. If effect of test chemical is more irritating, Q is higher. Calculation of "S Score". "S Score" is calculated using the most sensitive endpoint (endpoint can change from chemical to chemical). The scores recorded for the most sensitive endpoint are summarized for the 6 eggs. (Maximum for 6 eggs is 18)	Irritation Index calculated using the formula: (301-sec H)/300*5+(301-sec L)/300*7=(301-sec C)*9; where H=Hemorrhage, L=Vessel Lysis; C=Coagulation; sec=starting second	Not Noted

	Schlage et al. (1999)	Steiling et al. (1999)	Budai and Várnagy (2000)	Djabari et al. (2002)
Classification Scheme		**Q-SCORE: Slightly Irritating:** Q <= 0.8; **Moderately Irritating:** 0.8 < Q <1.2; **Irritating:** 1.2 <=Q<2.0; **Severely Irritating:** Q <= 2.0; **S-SCORE: Slightly Irritating** S < 6 ; **Moderately Irritating**: 6<= S <12; **Irritating**: 12 <= S ≥16; **Severely Irritating** S>=16	**Practically None**: 0-0.9; **Slight Irritation**: 1-4.9; **Moderate Irritation**: 5-8.9; **Strong Irritation**: 9-21	**Practically None**: 0-0.9; **Slight Irritation**: 1-4.9; **Moderate Irritation**: 5-8.9; **Strong Irritation**: 9-21
GLP Compliance?		Yes	Not Noted	Not Noted
Notes				Cites Luepke 1985 and Luepke and Kemper 1986 as basis for protocol and scoring scheme

Abbreviations: CAM = Chorioallantoic membrane, ECETOC = European Centre for Ecotoxicology and Toxicology of Chemicals, FHSA = Federal Hazardous Substances Act (1964), IS = Irritation score, IT = Irritation threshold concentration, NaCl = Sodium chloride, NaOH = Sodium hydroxide, PBS = Phosphate buffered saline, SDS = Sodium dodecyl sulfate, Sec = seconds, TSA = Test substance applicator.